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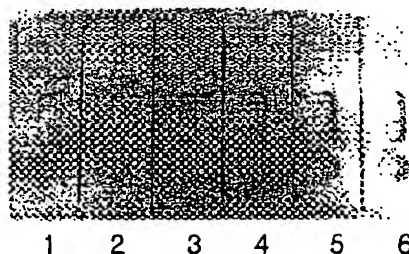
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(54) Title: MONOCLONAL ANTIBODY AGAINST ASIALO ALPHA 1-ACID GLYCOPROTEIN, IMMUNOCHROMATO-  
GRAPHIC STRIP COMPRISING THE MONOCLONAL ANTIBODY, AND METHOD FOR DIAGNOSING LIVER DISEASES  
USING THE IMMUNOCHROMATOGRAPHIC STRIP



1 :  $\alpha_1$ -acid glycoprotein + heptoglobin +  $\alpha_2$ -macroglobulin  
2 : normal person  
3 : 1.5  $\mu\text{g/ml}$   
4 : 2.0  $\mu\text{g/ml}$   
5 : 3.0  $\mu\text{g/ml}$   
6 : 4.0  $\mu\text{g/ml}$

(57) Abstract: The present invention relates to a method for diagnosing a liver disease rapidly in an early stage. More particularly, the present invention relates to a monoclonal antibody against asialo  $\alpha_1$ -acid glycoprotein; a method for diagnosing a liver disease which evaluates asialo  $\alpha_1$ -acid glycoprotein in a test sample by using said monoclonal antibody; and an diagnostic strip for immunochromatography composed of said monoclonal antibody against asialo  $\alpha_1$ -acid glycoprotein and Ricinus communis agglutinin (RCA). The diagnostic device of the present invention is convenient to measure the concentration of asialo  $\alpha_1$ -acid glycoprotein and to diagnose a liver disease rapidly.

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MONOCLONAL ANTIBODY AGAINST ASIALO ALPHA 1-ACID GLYCOPROTEIN,  
IMMUNOCHROMATOGRAPHIC STRIP COMPRISING THE MONOCLONAL ANTIBODY, AND  
METHOD FOR DIAGNOSING LIVER DISEASES USING THE  
IMMUNOCHROMATOGRAPHIC STRIP

5

#### TECHNICAL FIELD

The present invention relates to a method for diagnosing a liver  
10 disease rapidly in an early stage. More particularly, the present  
invention relates to a monoclonal antibody against asialo  $\alpha$ 1-acid  
glycoprotein; a method for diagnosing a liver disease which  
'evaluates asialo  $\alpha$ 1-acid glycoprotein in a test sample by using  
said monoclonal antibody; and an diagnostic strip for  
15 immunochromatography composed of said monoclonal antibody against  
asialo  $\alpha$ 1-acid glycoprotein and Ricinus communis agglutinin (RCA).  
The diagnostic device of the present invention is convenient to  
measure the concentration of asialo  $\alpha$ 1-acid glycoprotein and to  
diagnose a liver disease rapidly.

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#### BACKGROUND

Liver disease including hepatitis, liver cirrhosis, and

hepatocarcinoma is the most prevalent disease in Korea, Japan, Taiwan, China and other Southeast Asian countries. Presently, liver diseases have been diagnosed by evaluating the content of bilirubin or urobilinogen from patients' urine, by measuring the contents of glutamic-oxaloacetic transaminase (GOT), glutamic pyruvic transaminase, total bilirubin, albumin, lactic acid dehydrogenase and the like so as to analyze the changes of biochemical components in blood and by detecting an antigen from hepatitis B virus (HBV) or hepatitis C virus (HCV) or antibody against these viruses. Besides, liver cirrhosis can be diagnosed by alpha-feto protein (AFP) and carcinoembryonic antigen (CEA) test. However, liver is a complex organ due to various functions and is vitally specific not to reveal an abnormal state outwardly. Furthermore, an early diagnostic method has not been established yet and thus liver disease is often difficult to be treated, since it is diagnosed after severely worsen.

The present inventors have developed a marker which diagnoses a liver disease in a early stage clinically and reflects the severity of patient exactly and then manufactured a diagnostic kit. It has been disclosed in the patent application and the treatise that the marker be a remarkable agent for diagnosing a liver disease.

Precisely, the present inventors have demonstrated the diagnostic method and the diagnostic kit in Korean patent application

PCT/KR00/00840 (Aug. 1, 2000), US patent application 09/662,363 (Sept. 13, 2000) and Korean patent application 10-2000-0040609 (July 14, 2000), which exploits the sandwich ELISA method by using the specific antibody and lectin and measures asialo glycoprotein in blood. This techniques are confirmed to be recurrent and accurate and thus to be useful for diagnosing liver functions and to treat hepatic diseases.

It is reported that asialo glycoprotein represent the prognostic status of hepatic disease as a marker in blood serum (T. Sawamura et al., *Gastroenterology* 1981, 81: 527-533; T. Sawamura et al., *Gastroenterology* 1984, 87: 1217-1221). In addition, it is elucidated that asialo glycoprotein help to detect the status of hepatic cancer since the concentration is proportional to the severity in liver cancer (T. Sawamura et al., *Gastrologia Japonica* 1985, 20: 201-208).

Conventionally, the receptor against asialo glycoprotein is separated from human or other animal such as rabbit and mouse, purified and applied as a capture protein in order to measure the concentration of asialo glycoprotein. After it is labeled with radioactive substrates, the competitive radioactive assay and electro immunodiffusion are accomplished (J. S. Marshall et al., *J. Lab. Clin. Med.* 1978, 92: 30-37; N. Serbource-Goguel et al., *Hepatology* 1983, 3: 356-359).

Unfortunately, there are some problems. Above all, the receptor

against asialo glycoprotein is difficult to be obtained in a large scale, although the test kit needs a large amount of asialo glycoprotein. In case of competitive radioreceptor assay, it is dangerous to use radioactive substance and hard to prepare special facilities for treating waste material and the like. In cases of electroimmunodiffusion, it is complicated to analyze data quantitatively. Especially, the competitive assay is not suitable for general diagnostic kit since it lacks accuracy and recurrence.

10

#### BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which;

FIG. 1 depicts  $\alpha$ 1-acid glycoprotein (AGP) and desialylated  $\alpha$ 1-acid glycoprotein purified from blood plasma by performing sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE).

FIG. 2 depicts the monoclonal antibody against asialo  $\alpha$ 1-acid glycoprotein produced from the hybridoma cell line of the present invention by performing western blotting.

FIG. 3 depicts the monoclonal antibody prepared in the present invention by performing ELISA method, which reacts only with asialo  $\alpha$ 1-acid glycoprotein and excludes heptoglobin and  $\alpha$ 2-macroglobulin.

5

FIG 4a depicts a planar view of the diagnostic strip for immunochromatography prepared in the present invention.

FIG 4b depicts a front view of the diagnostic strip for  
10 immunochromatography prepared in the present invention.

FIG 5 depicts the result which is measured by using the cassette type diagnostic strip for immunochromatography prepared in the present invention and indicates asialo  $\alpha$ 1-acid glycoprotein in a  
15 concentration-dependent mode.

#### DISCLOSURE OF THE INVENTION

20 In order to settle above-mentioned technical problems and to diagnose a liver disease rapidly and easily by detecting asialo glycoprotein, the present inventors have attempted to develop a monoclonal antibody specific for asialo  $\alpha$ 1-acid glycoprotein (AsAGP), a diagnostic method for liver disease by using said

monoclonal antibody and a diagnostic strip for immunochromatography useful for the same method.

The object of the present invention is to provide a device and a method for detecting a liver disease easily.

5 In order to attain said object, the present invention provides a monoclonal antibody binding only with asialo  $\alpha$ 1-acid glycoprotein and excluding heptoglobin and  $\alpha$ 2-macroglobulin. The monoclonal antibody of the present invention also does not react asialo heptoglobin and asialo  $\alpha$ 2-macroglobulin. Preferably, the  
10 monoclonal antibody of the present invention is a subclass type IgG<sub>1</sub>.

The monoclonal antibody can be prepared by the process disclosed in the prior arts (Davidson R. L. and P. S. Gerald 1976, Improved techniques for the induction of mammalian cell hybridization by  
15 polyethylene glycol, Somatic Cell Genet., 2: 165~176; Knott C. L., Kuus-Reichel K., Liu R. and Wolfert R. L. 1997, Development of antibodies for diagnostic assays, In Price C. and Newman D. (eds.), Principles and Practice of Immunoassay, 2<sup>nd</sup> ed. New York, Stockton Press, 36~64; Gillete R. W. 1987, Alternatives to pristine priming  
20 for ascitic fluid and monoclonal antibody production, J. Immunol. Meth. 99, 21~23; Norwood T. H., C. J. Zeigler and G. M. Martin 1976, Dimethyl sulphoxide enhances polyethylene glycol-mediated somatic cell fusion, Somatic cell Genet., 2: 263~270) Precisely, it is manufactured by the process which comprises (1) separating



asialo  $\alpha$ 1-acid glycoprotein and (2) immunizing mice.

Above all, in order to produce the monoclonal antibody obtained above in a large scale, the hybridoma cell is prepared by the conventional process, separated, screened, injected into a mouse  
5 peritoneally and then collected from peritoneal fluid.

Concretely, asialo  $\alpha$ 1-acid glycoprotein is purified from blood by the process as described in the patent applications [PCT application PCT/KR00/00840 (Aug. 1, 2000), US patent application 09/662,363 (Sept. 13, 2000), Korean patent application 10-2000-  
10 0040609 (July 14, 2000)]. Asialo  $\alpha$ 1-acid glycoprotein is suspended by using phosphate buffer, blended by using Titer-MAX and applied to immunize a mouse. A spleen cell and a myeloma cell are separated from the experimental mouse immunized, fused and screened to select a hybridoma cell line specific for asialo  $\alpha$ 1-acid glycoprotein by ELISA method. In order to produce the  
15 monoclonal antibody specific for asialo  $\alpha$ 1-acid glycoprotein in a large scale from the hybridoma cell above, the hybridoma cell producing the monoclonal antibody against asialo  $\alpha$ 1-acid glycoprotein is injected to experimental mice and the peritoneal  
20 fluid of the mice containing the hybridoma cell in a high level is collected and separated a cell specific for the monoclonal antibody of the present invention.

In addition, the present invention also provides the hybridoma cell line which can produce in a large scale a monoclonal antibody

binding only with asialo  $\alpha$ 1-acid glycoprotein excluding heptoglobin and  $\alpha$ 2-macroglobulin.

In order to investigate whether the monoclonal antibody against asialo  $\alpha$ 1-acid glycoprotein obtained from the hybridoma cell be  
5 specific for asialo  $\alpha$ 1-acid glycoprotein or not, asialo  $\alpha$ 1-acid glycoprotein is first analyzed by performing electrophoresis and western blotting. It is further examined whether the monoclonal antibody of the present invention reacts only with asialo  $\alpha$ 1-acid glycoprotein and excludes other glycoproteins by performing ELISA  
10 method and the like as demonstrated in Example 2.

Preferably, the present invention provides the hybridoma cell line, producing the subclass type IgG<sub>1</sub> monoclonal antibody specific for asialo  $\alpha$ 1-acid glycoprotein and deposited to Korea Research Institute of Bioscience and Biotechnology, Gene Bank in May 24,  
15 2004 (accession number KCTC 10261 BP) under Budapest Treaty.

In addition, the present invention provides a method for detecting a hepatic disease which comprises steps (1) reacting a monoclonal antibody which binds only with asialo  $\alpha$ 1-acid glycoprotein and excludes heptoglobin and  $\alpha$ 2-macroglobulin; Ricinus communis  
20 agglutinin (hereinafter, referred to as "RCA") as a lectin, specifically recognizing asialo glycoprotein; and a test sample; and (2) measuring asialo  $\alpha$ 1-acid glycoprotein (AsAGP).

In the method for detecting a hepatic disease of the present invention, test samples can be analyzed on a microplate through a

sandwich enzyme immunoassay, an enzyme immunoassay onto an diagnostic strip for immunochromatography and various types of enzyme immunoassay. Especially, the enzyme immunoassay onto diagnostic strip for immunochromatography is the most convenient  
5 among these methods.

Preferably, the monoclonal antibody utilized in the diagnostic method of the present invention is a subclass type IgG<sub>1</sub> as described above. More preferably, the monoclonal antibody is produced from the mouse hybridoma cell line of the present  
10 invention deposited with accession number KCTC 10261 BP. Besides, as a lectin, RCA recognizing asialo  $\alpha$ 1-acid glycoprotein is preferable to be utilized.

The present invention provides a diagnostic strip for immunochromatography which comprises a monoclonal antibody binding  
15 only with asialo  $\alpha$ 1-acid glycoprotein and excluding heptoglobin and  $\alpha$ 2-macroglobulin; and lectin RCA recognizing asialo glycoprotein; measure the concentration of asialo  $\alpha$ 1-acid glycoprotein in a test sample; and diagnose a liver disease rapidly and easily.

20 Preferably, the present invention provides the diagnostic strip for immunochromatography which includes the monoclonal antibody As 16.89 deposited with the accession number KCTC 10261 BP and RCA as a lectin. The diagnostic strip for immunochromatography of the present invention comprises glass fiber (GF) membrane coated with

micro-particles such as gold-colloid conjugated with monoclonal antibody; nitrocellulose membrane (NC) in which RCA band is lined as a diagnostic line and an monoclonal antibody band as a standard line; a sample pad absorbing test sample solution; an absorbent  
5 pad discarding non-reactive substance; and an adhesive plastic backing for mounting the above-mentioned members.

The diagnostic strip for immunochromatography of the present invention is mounted in due turn, preferably NC membrane, GF membrane, a sample pad and absorbent pad are partially overlaid  
10 onto the adhesive plastic backing to transfer substance smoothly by capillary reaction.

The diagnostic strip for immunochromatography can be prepared by the conventional procedure. Preferably, the diagnostic strip of the present invention can be manufactured to a cassette type or a  
15 stick type.

Besides, the test sample is preferable to be blood or serum and 10-fold diluted by using elution buffer and the elution buffer is preferable to be 50 mM borate buffer containing 5% sucrose, 1% bovine serum albumin or 1% Triton X-100.

20 The diagnostic strip for immunochromatography of the present invention is used to detect a liver disease as follows: when a test sample is diluted and dropped onto the sample pad, both the standard line and diagnostic line are colored on the strip after 3 ~5 minutes so that the sample be judged according to colors

whether positive for hepatic disease or not. Precisely, if Ab-gold conjugate is adopted as a microparticle, the positive sample including asialo  $\alpha$ 1-acid glycoprotein reveals red color both on the standard line and the criteria line after a test and the  
5 negative sample including asialo  $\alpha$ 1-acid glycoprotein in a normal level (not a patient of liver disease) reveals red color only on the standard line.

As described above, the diagnostic strip for immunochromatography of the present invention can detect asialo  $\alpha$ 1-acid glycoprotein  
10 to reach a cutoff value, about 1.50  $\mu$ g/ml, which can diagnose a liver disease early and monitor its prognosis and treatment if the level of asialo  $\alpha$ 1-acid glycoprotein present in patients of liver cirrhosis and liver cancer continued to be evaluated.

Furthermore as illustrated above, the monoclonal antibody which  
15 reacts only with asialo  $\alpha$ 1-acid glycoprotein and excludes heptoglobin and  $\alpha$ 2-macroglobulin and the diagnostic strip for immunochromatography by using the same can inform the test result rapidly and conveniently so that liver diseases can be diagnosed easily. Therefore, it is expected to contribute to the prevention  
20 and treatment of liver diseases.

## EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrated as shown in the following Examples.

5 However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

10 <Example 1> Separation and purification of asialo  $\alpha$ 1-acid glycoprotein (AsAGP)

$\alpha$ 1-acid glycoprotein (AGP) was separated and purified from asialo glycoprotein contained in human blood plasma as follows.

2 NIH unit of thrombin was added to 200 ml of human blood plasma,  
15 stored at 37°C for 2 hours and at 4°C overnight and centrifuged to remove blood clot. The serum prepared above was dialyzed by using 0.05M of sodium acetate buffer (pH 4.3), loaded onto DEAE column previously equilibrated by using the same buffer, and then eluted through linear concentration gradient after 0.05 M of sodium  
20 acetate buffer (pH 4.3) and 0.1 M of sodium acetate buffer (pH 4.3) were mixed. Afterward, the optical density (OD) was measured at 280 nm and the data was illustrated in FIG. 1. The fractions including AGP and other proteins were collected, mixed with ammonium sulfate to 0.5 g/ml and centrifuged to precipitate

proteins. The resulting supernatant was mixed again with ammonium sulfate to 0.18 g/ml to precipitate proteins and the pellet was dissolved in a small amount of distilled water (D. W.), dialyzed sufficiently by using D. W. and then lyophilized.

5 40 mg of AGP separated above was hydrolyzed at 80°C for 2 hours by using 6 ml of 0.1 N sulfate solution, neutralized by using 1 N of sodium hydroxide and then dialyzed by using 0.01 N of phosphate buffer (pH 7.4). The desialylated  $\alpha$ 1-acid glycoprotein (AsAGP) prepared in the above procedure was loaded onto Sephadex G-200  
10 column, filtrated by using 0.01 M of phosphate buffer (pH 7.4) and calculated through OD value at 280 nm and finally the fractions including proteins were collected. The result was illustrated in FIG. 1: lane 1 is the standard marker of protein molecular weight; lane 2 is  $\alpha$ 1-acid glycoprotein; lane 3 and 4 are asialo  $\alpha$ 1-acid  
15 glycoprotein (AsAGP).

**<Example 2> Preparation of monoclonal antibody against asialo  $\alpha$ 1-acid glycoprotein**

20 **Immunization of mice**

In order to obtain an immunized mouse essential to prepare a hybridoma cell line producing a monoclonal antibody against asialo  $\alpha$ 1-acid glycoprotein, asialo  $\alpha$ 1-acid glycoprotein as an antigen was suspended well by using Titer-MAX, adjusted to 50  $\mu$ g/50 ml of

concentration and then injected into peritoneal cavity of Balb/c mice aged 6 ~ 8 weeks. After 2 weeks, the same amount of antigen mentioned in the first injection was mixed with Titer-MAX and injected repeatedly onto the same site. Through the same procedure, 5 the antigen was injected again after 7 days and repeatedly injected after 3 weeks. Afterward, the small amount of blood was collected from a tail of mouse and examined to evaluate a titer.

## (2) Cell fusion

10 In order to perform cell fusion for preparing a hybridoma cell line, mice were immunized with asialo  $\alpha$ 1-acid glycoprotein antigen mentioned above and spleen cells and myeloma cells were collected from the mice. Then,  $10^8$  of spleen cell and  $10^7$  myeloma cell (SP2/0) were washed sufficiently, mixed together and 1 ml of 15 PEG 1500 was poured for about 1 minutes, stirred slightly for about 1 minutes. Afterward, 9 ml of RPMI medium was added for about 3 minutes to reach 50 ml of final volume as stirred slowly. The cell suspension was centrifuged to collect the cell pellet, suspended again to  $1-2 \times 10^5$  cells/ml by using HAT medium, poured 20 into a 96-well microplate in 0.2 ml volume per well, and then incubated at 37°C in CO<sub>2</sub> incubator.

## Screening of hybridoma cell producing monoclonal antibody

In order to select a hybridoma cell specific for asialo  $\alpha$ 1-acid



glycoprotein, ELISA method was tried by exploiting a microplate coated with asialo  $\alpha$ 1-acid glycoprotein as follows. Precisely, asialo  $\alpha$ 1-acid glycoprotein antigen was put into a microplate in 100  $\mu$ l (1  $\mu$ g/ml) per well to coat the surface and washed off to  
5 remove antigens not reacted. The culture medium of hybridoma cell was poured to each well in 100  $\mu$ l, reacted for 2 hours and washed off by using Tween 20 phosphate buffer (PBST) to remove the culture medium not reacted. Afterward, goat anti-mouse IgG-horseradish peroxidase (HRP) was added, reacted for an hour at  
10 room temperature and washed off by using PBST solution. Then, ortho-phenylenediamine (OPD) was utilized as a substrate of peroxidase, reacted and examined to measure OD value at 490 nm with ELISA reader.

As a result, the cell line secreting antibodies highly binding to  
15 asialo  $\alpha$ 1-acid glycoprotein antigen was first screened and repeatedly selected to separate the hybridoma cell secreting a monoclonal antibody specific for asialo  $\alpha$ 1-acid glycoprotein antigen. The resulting cells were treated by the limiting dilution to become a monoclonal and the hybridoma cell line producing a  
20 monoclonal antibody was sorted again. Afterward, 2 clones having the highest titer such as asialo  $\alpha$ 1-acid glycoprotein 1 and asialo  $\alpha$ 1-acid glycoprotein 2 were collected, tried to measure the titer of supernatant in culture medium through ELISA method and then the subunit type of the monoclonal antibody was analyzed

by the double immunodiffusion. Consequently, the antibodies secreted from the clones of the present invention were identified as IgG<sub>1</sub> and IgM respectively.

The cell line secreting IgG<sub>1</sub> has been named as AS 16.89 and deposited to Korea Research Institute of Bioscience and Biotechnology, Gene Bank in May 24, 2004 (accession number KCTC 10261 BP).

#### Large scale production and purification of monoclonal antibody

10 In order to produce a monoclonal antibody from a hybridoma cell in a large scale, 0.5 ml of pristane was injected into peritoneal cavity of Balb/c mice. After 1 week, each cell line obtained in the above procedure (3) was injected to the experimental mice in 5 x 10<sup>6</sup> cells per mouse and then peritoneal fluid was collected from 15 the peritoneal cavity if swollen. The peritoneal fluid was centrifuged at 12,000 rpm to collect cells and the resulting supernatant was filtrated through protein G column to separate and purify the monoclonal antibody against asialo  $\alpha$ 1-acid glycoprotein and also stored at -20°C for the next experiments.

20

#### Identification of specificity of monoclonal antibody by western blot

In order to elucidate the property of monoclonal antibody specific for asialo  $\alpha$ 1-acid glycoprotein, the monoclonal antibody against

asialo  $\alpha$ 1-acid glycoprotein purified through the above-mentioned procedure (4) was identified by performing SDS-polyacrylamide gel electrophoresis and western blotting (See FIG 2). The western blotting was accomplished by a typical procedure in the prior arts.

5 In FIG. 2, lane 1 is a standard marker of protein molecular weight; lane 2,  $\alpha$ 1-acid glycoprotein; lane 3, asialo  $\alpha$ 1-acid glycoprotein; lane 4,  $\alpha$ 2-macroglobulin; and lane 5, heptoglobin.

10 Identification of specificity of monoclonal antibody by enzyme immunochemical assay

Asialo  $\alpha$ 1-acid glycoprotein, heptoglobin,  $\alpha$ 2-macroglobulin antigen were added to 96-well microplate in 100  $\mu$ l (1  $\mu$ g/ml) per well respectively, attached on the surface, reacted for 2 hours with the monoclonal antibody against asialo  $\alpha$ 1-acid glycoprotein as added in 100  $\mu$ l, and washed off by using phosphate buffer-Tween 15 20 (PBST) to remove the culture medium not reacted. Further, goat anti-mouse IgG-HRP was added, reacted for an hour at room temperature and washed sufficiently by using PBST solution. Afterward, ortho-phenylenediamine (OPD) was added as a substrate 20 of peroxidase and reacted so as to measure OD value at 490 nm with ELISA reader. As a result, it is confirmed that the monoclonal antibody against asialo  $\alpha$ 1-acid glycoprotein purified above be the monoclonal antibody specific for asialo  $\alpha$ 1-acid glycoprotein (See FIG. 3). In FIG. 3, AGP depicts  $\alpha$ 1-acid glycoprotein and

AsAGP, asialo  $\alpha$ 1-acid glycoprotein of the present invention.

<Example 3> Detection of liver disease by diagnostic kit for immunochromatography

5

The diagnostic kit for immunochromatography of the present invention which can measure the concentration of asialo  $\alpha$ 1-acid glycoprotein in a test sample by the immune reaction between the monoclonal antibody As 16.89 specific for asialo  $\alpha$ 1-acid glycoprotein produced above and asialo  $\alpha$ 1-acid glycoprotein in blood was manufactured and applied in clinical fields as follows.

Preparation of diagnostic kit for immunochromatography

15 The diagnostic kit for detecting asialo  $\alpha$ 1-acid glycoprotein in blood is composed of following components as described below.

monoclonal antibody (As16.89) specific for asialo  $\alpha$ 1-acid glycoprotein fixed onto solid carrier such as microplate lectin (RCA)-horseradish peroxidase (HRP) against asialo  $\alpha$ 1-acid glycoprotein

20 sample dilution buffer (1% BSA/PBST)

enzyme substrate solution (OPD)

washing buffer (PBST)

standard solution of asialo  $\alpha$ 1-acid glycoprotein

stopping buffer

### Measurement

- a. The standard solution (1  $\mu\text{g/ml}$ ) of asialo  $\alpha$ 1-acid glycoprotein was poured in 100  $\mu\text{l}$  respectively to microplate on which the monoclonal antibody As 16.89 was fixed. Experiment  
5 samples, including 40 normal persons, 155 patients without liver disease, 36 patients of acute hepatitis, 272 patients of chronic hepatitis (CH), 230 patients of liver cirrhosis, 72 patients of hepatocarcinoma (HCC) proceeded from liver cirrhosis, were obtained from St. Mary Hospital, Catholic Medical School. The  
10 blood sample was diluted to 1 : 10 ratio by using the dilution buffer, allotted onto each well of microplate in 100  $\mu\text{l}$  and incubated for 120 minutes at room temperature.
- b. After reacted, the washing buffer was added in 100  $\mu\text{l}$  per well and washed three times repeatedly.
- 15 c. The lectin (RCA)-HRP diluted by the process for the preparation was allotted onto each well of microplate and reacted for 60 minutes at room temperature.
- d. Step b was repeated.
- e. The enzyme substrate solution (OPD) was allotted onto each well  
20 of microplate above.
- f. The stopping buffer was added on each well in 100  $\mu\text{l}$  to stop the enzymatic reaction.
- g. OD value was measured at 490 nm with ELISA reader.
- As a result, the content of asialo  $\alpha$ 1-acid glycoprotein in blood

was evaluated through the above-mentioned procedure as illustrated in Table 1.

<Table 1>

Measurement of blood AsAGP

5 (cutoff value: 1.50  $\mu\text{g/ml}$ )

class		sum	Ave. +SD $\mu\text{g/ml}$ *	AsAGP > 1.50 $\mu\text{g/ml}$		AsAGP > 1.50 $\mu\text{g/ml}$	
				No. of sample	%	No. of sample	%
standard group	sum	196	0.89 X 0.46	19	10. 0	177	90
	normal	41	0.84 X 0.41	2	5	39	95
	non- hepatic disease	155	0.90 X 0.48	17	11	138	89
Group 1 of liver disease	acute hepatitis	36	1.33 X 0.77	9	25	27	75
Group 2 of liver disease	sum	574	2.48 X 0.89	323	56	251	44
	chronic hepatitis (CH)	272	1.63 X 0.45	99	36	173	64
	liver cirrhosis (LC)	230	3.12 X 0.42	165	72	65	28
	hepatocarc inoma (HCC) with liver cirrhosis (LC)	72	3.64 X 0.1	59	82	13	18

Consequently, it is confirmed that the concentration of asialo  $\alpha$ -1-acid glycoprotein (AsAGP) for normal persons and patients of non-hepatic disease be below 1.00  $\mu\text{g/ml}$  in an average level and the number of sample over 1.50  $\mu\text{g/ml}$  in the concentration of AsAGP be about 10%. In patient groups of acute hepatitis, chronic hepatitis, liver cirrhosis and hepatocarcinoma with liver cirrhosis, the blood level of AsAGP was evaluated to average 1.33  $\mu\text{g/ml}$ , 1.63  $\mu\text{g/ml}$ , 3.12  $\mu\text{g/ml}$  and 3.64  $\mu\text{g/ml}$  respectively. The concentrations be remarkably distinguished from those of standard group, even if the ratio of sample over 1.5  $\mu\text{g/ml}$  of AsAGP be 25%, 36%, 72% and 82% respectively. Therefore, it is verified that this test be effective upon the diagnosis of hepatic diseases.

Hence, according to data demonstrated above, the cutoff value for diagnosing a liver disease was decided to be 1.50  $\mu\text{g/ml}$  when the monoclonal antibody As 16.89 of the present invention was used for the enzyme immunoassay.

#### <Example 4> Preparation of diagnostic strip for immunochromatography

20

##### Preparation of monoclonal antibody-gold conjugate

The monoclonal antibody specific for asialo  $\alpha$ -1-acid glycoprotein selected in the present invention was added in 15  $\mu\text{g/ml}$  to the colloidal solution of gold particles, reacted for 2 hours at room

temperature and rotated. Then, 10% of BSA was blended in 1/10 volume to become 1% of concentration and again reacted for an hour to prepare Ab-gold conjugate. Afterward, the resultant was centrifuged at 12,000 rpm for 40 minutes to discard the supernatant solution and 2 mM of borate buffer was added to wash off Ab-gold conjugate. The resultant was washed repeatedly, three times and finally 2 mM of borate buffer containing 1% BSA was added in about 1/10 volume of gold solution and suspended. After measured with UV spectrometer, the OD value at 530 nm was adjusted to 3.00 by dilution.

#### Sample pad

The sample pad is a member absorbing test sample and is made of cellulose material for this purpose.

#### Glass fiber (GF) membrane

The glass fiber is a component on which AsAGP in test sample and the monoclonal antibody prepared by the process of the present invention are reacted. The GF membrane was coated with gold colloid particle- As 16.89 on the surface and prepared as follows. The monoclonal antibody produced from hybridoma cell line As 16.89 of the present invention was conjugated onto the membrane as illustrated in step (1).

GF membrane (1.0 cm X 0.7 cm) purchased from Millipore Co. Ltd.



was soaked by using 20 mM sodium borate buffer, sprayed uniformly by using gold colloid particle- As 16.89 onto the surface, dried at 37°C and thus made to GF membrane pad for immunochromatography (also conjugate pad) on which a monoclonal antibody conjugated  
5 with a coloring particle is fixed.

#### Nitrocellulose (NC) membrane and lining

Nitrocellulose membrane purchased from Millipore Co. Ltd. was cut to a proper size (0.7 cm X 5 cm), lined at a point of about 3.4 cm  
10 from the bottom of plastic backing with goat anti-mouse IgG as a standard line and at a point of 2.7 cm as a criteria line for detecting asialo  $\alpha$ 1-acid glycoprotein by using RCA purchased from EY Lab, and then dried to complete the NC membrane.

#### 15 Absorbent pad

Cellulose membrane was used to absorb substance not reacted in a test sample after an immune reaction and to transfer the sample solution through capillary reaction.

#### 20 Preparation of diagnostic strip for immunchromatography

As illustrated in FIG. 4a and FIG. 4b, the above-mentioned members were mounted onto the adhesive plastic backing. Precisely, NC membrane, GF membrane, sample pad and absorbent pad were arranged in due turn, overlaid evenly in about 0.1 cm portion and fixed by

adhesion in order to transfer substance smoothly through capillary reaction.

5     <Example 5> Analysis of asialo  $\alpha$ 1-acid glycoprotein by using  
diagnostic strip for immunochromatography

10     Serum sample was diluted to 1 : 10 ratio by using elution buffer (such as 50 mM borate buffer containing 5% sucrose, 1% bovine serum albumin or 1% Triton X-100) and added in 60 ~ 70  $\mu$ l volume onto the sample pad of diagnostic strip for immunochromatography prepared in Example 4 so as to examine the standard line and the criteria line after 3 ~ 5 minutes whether colored or not.

15     FIG. 5 depicts the result from the cassette type strip for immunochromatography. NO. 1 is the standard dropping the mixture of  $\alpha$ 1-acid glycoprotein (AGP), heptoglobin and  $\alpha$ 2-macroglobulin; NO. 2, the diagnostic strip in the serum of normal person; NO. 3 ~ NO. 6, the strip in 1.5  $\mu$ g/ml, 2.0  $\mu$ g/ml, 3.0  $\mu$ g/ml and 4.0  $\mu$ g/ml of asialo  $\alpha$ 1-acid glycoprotein respectively. As illustrated in  
20     FIG. 5, in NO. 3, the line is obscure; and in NO. 4 ~ NO. 6, red colors are clear both on the standard line (upper) and the criteria line (lower).

The diagnostic strip for immunochromatography of the present invention is observed that only the standard line become red in

the test sample of normal person (negative) under the cutoff value, 1.50  $\mu\text{g/ml}$  of asialo  $\alpha$ 1-acid glycoprotein since antigen-antibody-enzyme conjugate complex was not formed. On the other hand, when asialo  $\alpha$ 1-acid glycoprotein level increased to more than 1.50  $\mu\text{g}$  /ml due to a liver disease, both the lines become red, in which asialo  $\alpha$ 1-acid glycoprotein was absorbed on the sample pad, reacted with gold particle-monoclonal antibody As 16.89 conjugate on GF membrane to form an immune complex, transferred onto NC membrane through capillary reaction and conjugated with RCA in the criteria line on NC membrane to make gold precipitate. Furthermore, the criteria line can be varied in thickness and color density, depending upon the concentration of asialo  $\alpha$ 1-acid glycoprotein. Therefore, it is possible to predict the severity of liver disease by the concentration of asialo  $\alpha$ 1-acid glycoprotein in the test sample.

#### INDUSTRIAL APPLICABILITY

As illustrated above, the monoclonal antibody specific for asialo  $\alpha$ 1-acid glycoprotein and the diagnostic strip and the diagnostic kit for immunochromatography by using the same of the present invention can be utilized to evaluate asialo  $\alpha$ 1-acid glycoprotein present in blood sample rapidly and easily. Therefore, the

monoclonal antibody and the diagnostic device of the present invention can monitor the severity, the treatment and the prognosis of liver diseases efficiently.

- 5 Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention.
- 10 Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT  
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

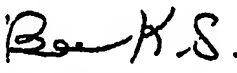
## INTERNATIONAL FORM

## RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

Issued pursuant to Rule 7.1

TO : Kobias

BVC 115, Korea Research Institute of Bioscience and Biotechnology,  
#52, Oun-dong, Yusong-ku, Taejeon 305-333,  
Republic of Korea

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR:  As16.29 (lymphoblast-like hybridoma cell lines)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  KCTC 10281BP
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I above was accompanied by: <input checked="" type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on May 24 2002.	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____	
<b>V. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
Name: Korean Collection for Type Cultures  Address: Korea Research Institute of Bioscience and Biotechnology (KRIIB) #52, Oun-dong, Yusong-ku, Taejeon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):    BAE, Kyung Seok, Director Date: June 18 2002

Form D/W (KCTC Form 17)

date: 2002

What is claimed is:

1. A monoclonal antibody which reacts only with asialo  $\alpha$ 1-acid glycoprotein and excludes heptoglobin and  $\alpha$ 2-macroglobulin.

5

2. The monoclonal antibody according to claim 1, which is a subclass type IgG<sub>1</sub>.

3. A cell line which produces in a large scale a monoclonal antibody reacting only with asialo  $\alpha$ 1-acid glycoprotein and excluding heptoglobin and  $\alpha$ 2-macroglobulin.

10

4. The cell line according to claim 3, which is prepared by fusing a spleen cell and a myeloma cell extracted from a mouse immunized with asialo  $\alpha$ 1-acid glycoprotein.

15

5. The cell line according to claim 4, which produces the subclass type IgG<sub>1</sub> monoclonal antibody specific for asialo  $\alpha$ 1-acid glycoprotein and is deposited to Korea Research Institute of Bioscience and Biotechnology, Gene Bank in May 24, 2004 (accession number KCTC 10349 BP) under Budapest Treaty.

20

6. A method for diagnosing a liver disease in which a monoclonal antibody which reacts only with asialo  $\alpha$ 1-acid

glycoprotein and excludes heptoglobin and  $\alpha$ 2-macroglobulin; and lectin RCA (*Ricinus communis* agglutinin) recognizing asialo glycoprotein are reacted with a test sample to measure the amount of asialo  $\alpha$ 1-acid glycoprotein (AsAGP).

5

7. The method for diagnosing a liver disease according to claim 6, in which the monoclonal antibody is the subclass IgG<sub>1</sub> produced from the mouse cell line deposited with the accession number KCTC 10261 BP.

10

8. The method for diagnosing a liver disease according to claim 6, in which the test sample is blood or serum.

15

9. An diagnostic strip for immunochromatography which comprises a monoclonal antibody reacting only with asialo  $\alpha$ 1-acid glycoprotein excluding heptoglobin and  $\alpha$ 2-macroglobulin; and RCA recognizing asialo glycoprotein as a lectin; and reacts a test sample at over 1.50  $\mu$ g/ml of asialo  $\alpha$ 1-acid glycoprotein to detect a liver disease.

20

10. The diagnostic strip for immunochromatography according to claim 9, in which the monoclonal antibody is As 16.89 deposited with the accession number KCTC 10261 BP.

11. The diagnostic strip for immunochromatography according to claim 9, in which the monoclonal antibody is conjugated with a micro-particle such as colloid type gold particle.

5 12. The diagnostic strip for immunochromatography according to claim 11, which comprises (1) glass fiber (GF) coated with micro-particles conjugated with the monoclonal antibody; (2) nitrocellulose membrane (NC) including a standard line and a diagnostic line to detect a glycoprotein; (3) a sample pad for a  
10 test sample; (4) a absorbent pad for non-reactive substance; and (4) an adhesive plastic backing for mounting above-mentioned members.

13. The diagnostic strip for immunochromatography according to  
15 claim 12, in which NC membrane, GF membrane, a sample pad and a absorbent pad are partially overlaid on said adhesive plastic backing in due turn to transfer substance by capillary reaction and an RCA band as a diagnostic line and an antibody band as a standard line are separated in some interval.

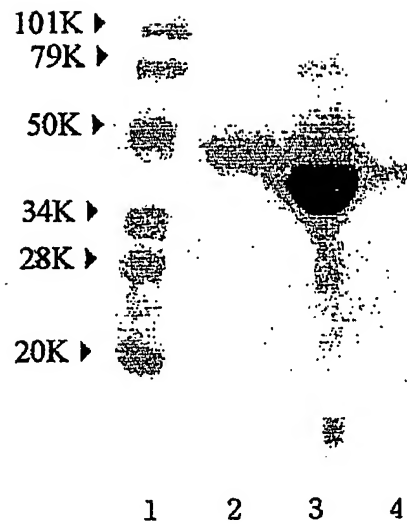
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14. The diagnostic strip for immunochromatography according to claim 9, in which said test sample is blood or serum 10-folds diluted by using an elution buffer.

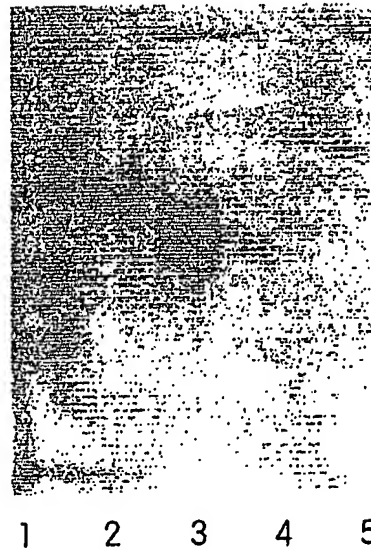


15. The diagnostic strip for immunochromatography according to claim 14, in which said elution buffer is 50 mM of borate buffer containing 5% sucrose, 1% bovine serum albumin or 1% Triton X-100.
- 5 16. The diagnostic strip for immunochromatography according to claim 9, in which a standard line and a diagnostic line are colored on said strip to indicate positive for a hepatic disease.
17. The diagnostic strip for immunochromatography according to claim 9, which is a cassette type.
- 10 18. The diagnostic strip for immunochromatography according to claim 9, which is a stick type.
- 15 19. A diagnostic kit for immunochromatography which comprises a monoclonal antibody reacting only with asialo  $\alpha$ 1-acid glycoprotein and excluding heptoglobin and  $\alpha$ 2-macroglobulin; RCA (Ricinus communis agglutinin) recognizing asialo glycoprotein as a lectin; and reacts a test sample at over 1.50  $\mu$ g /ml of asialo  $\alpha$ 1-acid glycoprotein to detect a liver disease.
- 20 20. The diagnostic kit for immunochromatography according to claim 19, in which the monoclonal antibody is As 16.89 deposited with the accession number KCTC 10261 BP.

1/3



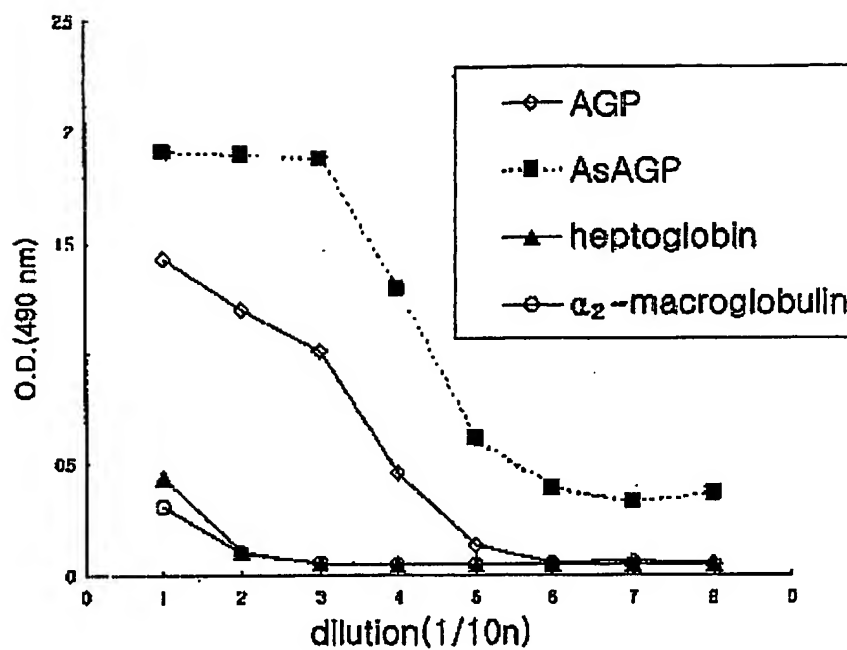
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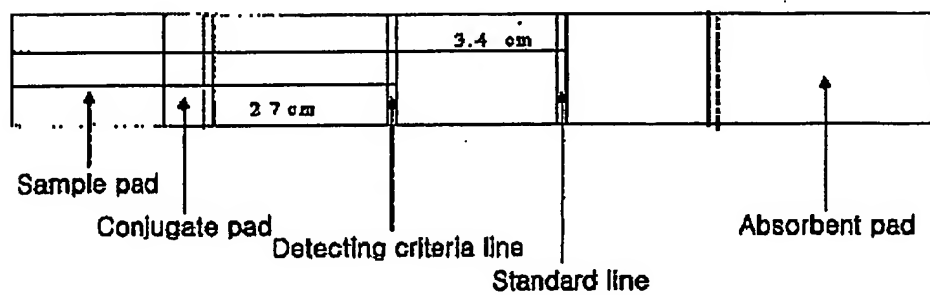
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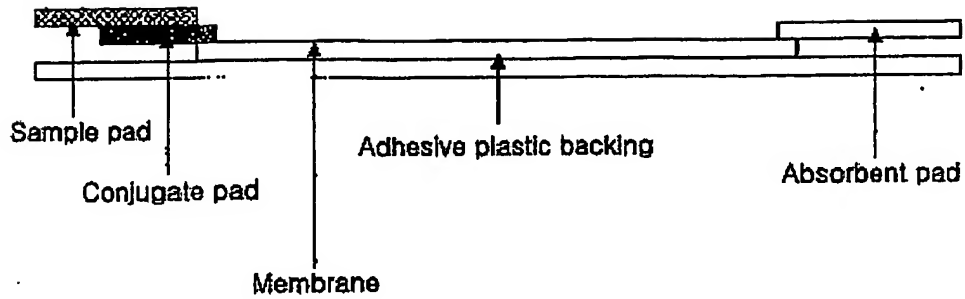
도 3



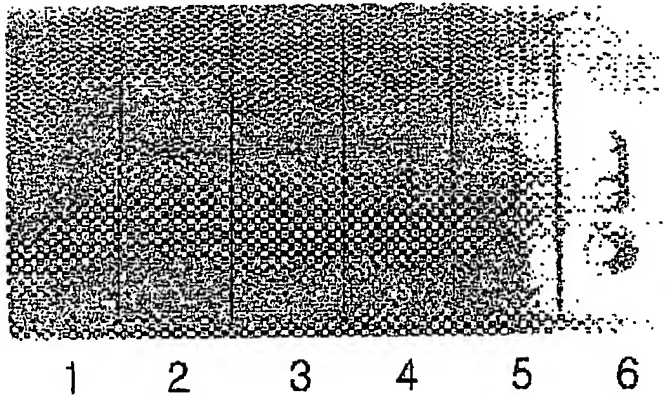
도 4a

10/540848

3/3



도 4b





- 1 :  $\alpha_1$ -acid glycoprotein + heptoglobin +  $\alpha_2$ -macroglobulin  
 2 : normal person  
 3 : 1.5  $\mu\text{g/ml}$   
 4 : 2.0  $\mu\text{g/ml}$   
 5 : 3.0  $\mu\text{g/ml}$   
 6 : 4.0  $\mu\text{g/ml}$

도 5

## INTERNATIONAL SEARCH REPORT

.....national application No.  
PCT/KR2003/002860

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC7 C07K 16/18, C07K 16/34		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
IPC7 C07K 16/18, C07K 16/34, G01N 33/68		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Korean Patents and applications for inventions since 1975		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
NCBI PubMed, Esp@cenet, CA "asialo alpha 1-acid glycoprotein, monoclonal antibody, kit, immunochromatographic strip"		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2001/035105 A1 (KRIBB & KOBIA CO., LTD.) 17 May 2001 See the whole document	1-8, 19, 20
Y	EP 0199196 A2 (KURARAY CO., LTD.) 29 October 1986 See the whole document	1-8, 19, 20
A	WO 1998/022824 A1 (ZER AVRAHAM & ZER TAMAR) 28 May 1998 See the whole document	9-18
A	DILL K. & BEARDEN D.W., 'Detection of human asialo-alpha(1)-acid glycoprotein using a heterosandwich immunoassay in conjunction with the light addressable potentiometric sensor', In: Glycoconj. J., 1996, Vol. 13(4), pp. 637-641. See the abstract	1-20
A	LUNDY F.T. & WISDOM G.B., 'An antibody-lectin sandwich assay for quantifying protein glycoforms', In: Mol. Biotechnol., 1999, Vol. 12(2), pp. 203-206. See the abstract	1-20
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 12 MAY 2004 (12.05.2004)		Date of mailing of the international search report 12 MAY 2004 (12.05.2004)
Name and mailing address of the ISA/KR  Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140		Authorized officer CHO, YOUNG GYUN Telephone No. 82-42-481-8132 

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2003/002860

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 6-8  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 6-8 are directed to diagnostic methods of the human or animal body, the search has been carried out, based on the alleged effects of the monoclonal antibody and the composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any addition fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

PCT/KR2003/002860

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2001/035105 A1	17.05.2001	KR 2001/049795 A AU 6187300 A	15.06.2001 06.06.2001
EP 0199196 A2	29.10.1986	US 4894442 A JP 62044199 A	16.01.1990 26.02.1987
WO 1998/022824 A1	28.05.1998	AU 4963897 A	10.06.1998

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